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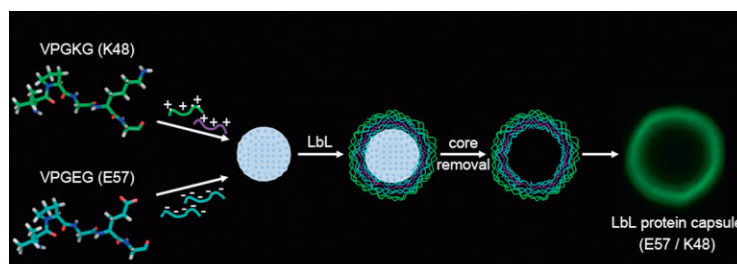
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De Novo Design of Supercharged, Unfolded Protein Polymers, and Their Assembly into Supramolecular Aggregates^a

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Here we report for the first time the design and expression of highly charged, unfolded protein polymers based on elastin-like peptides (ELPs). Positively and negatively charged variants were achieved by introducing lysine and glutamic acid residues, respectively, within the repetitive pentapeptide units. Subsequently it was demonstrated that the monodisperse protein polyelectrolytes with precisely defined amino acid compositions, sequences, and stereochemistries can be transferred into superstructures exploiting their electrostatic interactions. Hollow capsules were assembled from oppositely charged protein chains by using the layer-by-layer technique. The structures of the capsules were analyzed by various microscopy techniques revealing the fabrication of multilayer containers. Due to their low toxicity in comparison to other polyelectrolytes, supercharged ELPs are appealing candidates for the construction of electrostatically induced scaffolds in biomedicine.



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^a Supporting information for this article is available at the bottom of the article's abstract page, which can be accessed from the journal's homepage at <http://www.mrc-journal.de>, or from the author.

^b Shared first authorship due to equal contribution.

Introduction

Genetically encoded polypeptides with repetitive motifs have gained increasing attention in recent years due to their high potential for biotechnological and biomedical applications. This development was mainly fueled by progress in recombinant DNA technology allowing precise control of the structure of the resulting macromolecules.^[1,2] Important examples are silk-like,^[3] collagen-like^[4,5], and elastin-like proteins (ELPs).^[6] The latter are derived from a repeating motif within a hydrophobic domain of mammalian tropoelastin. The most common pentapeptide motif has the sequence (VPGXG)_n with X being any guest amino acid except proline and *n* denoting the number of repeats.^[7] The structural and physical properties of ELPs, such as their elastic/mechanical as well as thermoresponsive behavior, have been investigated.^[8–10] Their ability to undergo a reversible phase transition at the so-called lower critical

solution temperature (LCST) has been exploited for the purification of proteins^[11] and DNA.^[12] For tissue engineering purposes, ELPs were designed as thermally sensitive hydrogels that solidify when injected into the body.^[13] Furthermore, their temperature responsiveness was utilized for drug delivery applications. In hyperthermia treatment, ELPs were accumulated in tumors^[14] and the LCST-behavior was employed to induce micelle formation of block ELP structures.^[15]

Results and Discussion

Design, Preparation, and Characterization of Elastin-like Polypeptides (ELPs)

The choice of different guest amino acids within the ELP motif allows the precise control of LCST and the incorporation of chemical modifications.^[16] Here, we took advantage of the flexibility of amino acid composition at the fourth position within the repeat to transform ELPs into unprecedented highly charged anionic and cationic polyelectrolytes. These structures of biosynthetic origin are much better defined than their chemically synthesized counterparts. To assess their viability in a common application for polyelectrolytes in a biomedical context, these materials were transformed into superstructures, i.e., hollow capsules, employing the electrostatic interactions of oppositely charged variants.

We thus decided to introduce lysine and glutamic acid residues in order to obtain highly positively and negatively charged polypeptide chains, respectively. Monomer units of the ELP gene encoded ten pentapeptide repeats (Val-Pro-Gly-Lys/Glu-Gly) and were multimerized using recursive directional ligation, as described by Meyer and Chilkoti.^[10] ELPs with 48 positive (K48) or 57 negative (E57) charges were produced in *E. coli* and purified (Supporting Information). Protein yields were 1 and 5 mg per liter of bacterial cell culture for K48 and E57, respectively. The purity of the products was analyzed by polyacrylamide gel electrophoresis and subsequent staining with either SimplyBlueTM SafeStain (Invitrogen) or copper(II) chloride (Figure 1). ELPs exhibited reduced electrophoretic mobilities compared to globular proteins, a finding widely observed with ELPs.^[10] It was also observed that the negatively charged variant E57 was poorly stained with the SimplyBlue SafeStain as well as with Coomassie brilliant blue R-250 (data not shown). Staining with copper(II) chloride, however, led to a clear E57 protein band and a white K48 protein band against an opaque, whitish-blue background (Figure 1b). Analysis of the purified ELPs by mass spectrometry resulted in molecular weights that were in excellent agreement with the theoretical values (see Supporting Information). As expected,^[17] the proteins K48 and E57 do not exhibit LCST

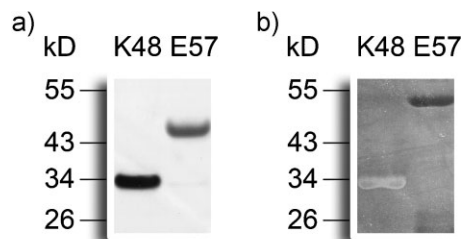


Figure 1. Purified ELP variants K48 and E57 examined by SDS-PAGE. (a) 4–12% NuPAGE[®] gel stained with SimplyBlueTM safe stain. K48: 2 μ g, E57: 8 μ g. (b) 12% SDS-PAGE gel stained with a 0.3 M copper (II) chloride solution. K48: 2 μ g, E57: 4 μ g.

behavior until 90 °C and show mainly random coil structure when dissolved in water (see Supporting Information).

Capsule Preparation and Characterization

After successful expression our next goal was to exploit the high net charges of K48 and E57 for self assembly of the ELP variants into supramolecular structures, namely multilayer polypeptide capsules, using a layer-by-layer (LbL) technique^[18] (Figure 2). This technique is based on the consecutive assembly of oppositely charged polymers around a preformed charged spherical template^[18] with typical diameter from a few hundred nm to a few μ m. At the end of the LbL adsorption process, the cores can be successfully removed to obtain hollow and intact capsules. Polymer containers based on the LbL technique have recently attracted high interest for a variety of different applications, ranging from drug delivery systems and targeted gene therapy to biosensor devices.^[19,20] To date, capsules have been made of synthetic and biodegradable polyelectrolytes,^[21,22] comprising natural molecules such as oligonucleotides^[23] and proteins,^[24,25] which demonstrates the high versatility of LbL assembly.

Using standard LbL preparation techniques^[18] and employing supercharged proteins E57 and K48 as building

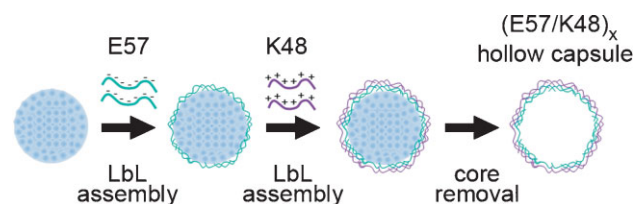


Figure 2. Schematic illustration of the multiple assembly of the two oppositely supercharged proteins onto spherical CaCO_3 porous microparticles via LbL assembly technique, and fabrication of a hollow protein-based capsule by dissolution of the template core. The turquoise lines represent the supercharged negative E57 ELP, the violet lines represent the supercharged positive K48 ELP. For simplicity, only two layers are shown. Capsules are not drawn to scale.

blocks, we generated capsules exhibiting the following wall structure: (DEXS/pARG)(E57/K48)₃(E57/K48_{AF488})E57, where DEXS denotes dextran sulfate and pARG poly-(L-arginine), two charged biodegradable polymers made from naturally occurring monomers (see Supporting Information). The use of DEXS/pARG as a first bi-layer proved crucial to growing a stable multilayer (E57/K48) wall, providing a barrier against diffusion of the proteins into the porous CaCO₃ particle templates (see Supporting Information, Figure S6). For visualization purposes, one layer of K48 was fluorescence labeled with Alexa AF488 (see Supporting Information). Capsules were characterized with confocal laser scanning microscopy (CLSM), transmission electron microscopy (TEM), and scanning electron microscopy (SEM).

In Figure 3 representative images of the capsules formed by supercharged proteins are shown. These images clearly demonstrate the existence of capsules with an empty interior and stable walls. Notably, the dissolution of the core is a critical step in the preparation of hollow capsules, as it may result in capsules breaking or swelling due to decomposition conditions (i.e., low pH) where the polymer wall may disaggregate. In view of that, the results reported in Figure 3 indicate that a hollow and stable wall remains when the inorganic core is dissolved. Compared to capsules formed by standard synthetic polyelectrolytes, such as polystyrene sulfonate (PSS) and poly(allylamine hydrochloride) (PAH),^[18] the walls of capsules based on supercharged proteins are rather porous (see Supporting Information, Figure S10, 12–13). This may be a result of the lower charge density of E57 and K48 compared to PSS and PAH (see Supporting Information, Table 1), which results in a higher mechanical pressure during the dissolution procedure. For instance, permeability tests performed on (E57/K48) capsules without cores showed the diffusion across the protein wall of the encapsulated dextran (500 kDa MW) (see Supporting Information, Figure

S10), thus indicating the formation of large pores in the multilayer wall. This hypothesis was subsequently supported by TEM and SEM observation of capsules after core dissolution (see Supporting Information, Figure S12,13). At any rate, the two structural compartments of capsules, cavity, and wall, are well defined and prove successful and stable assembly (Supporting Information, Figure S7 and S9).

Evaluation of in vitro Cytotoxicity

Such capsules might be appealing containers for use in biomedicine. Since positively charged polymers are the most common source of toxicity in charged systems due to their interaction with anionic intracellular components,^[26–28] the toxicity of K48 was investigated and compared to the other positive polyelectrolytes used for the synthesis of capsule controls (i.e., PLL, PAH, pARG). A fluorimetric metabolic assay employing NIH/3T3 embryonic fibroblast cells was utilized to assess cytotoxicity. The normalized fluorescence of Resorufin, a dye indicating metabolically active cells, was plotted against polyelectrolyte concentrations (Figure 4). The resulting dose response curves yielded the following polymer concentrations causing 50% cell death (LD50, mg · mL⁻¹) in decreasing order of toxicity: PLL (LD50 = 0.007), PAH (LD50 = 0.009), pARG (LD50 = 0.015), and K48 (LD50 = 0.196). PLL, PAH, and pARG exhibited similar dose response curves with similar LD50 values, whereas the curve for K48 was strongly shifted to higher concentration values. This indicates that lower concentrations of PLL, PAH, and pARG than of K48 are able to induce cell death. K48 induces toxicity at the maximum concentration value used (1 mg · mL⁻¹) and this effect was immediately mitigated by halving the dose. A plateau level of viability was reached at a concentration of 3.1×10^{-2} mg · mL⁻¹.

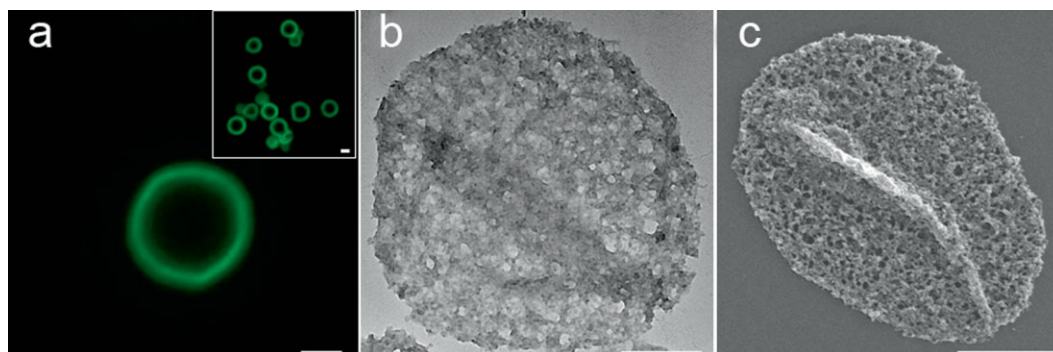


Figure 3. Structural analysis of capsules assembled by the supercharged proteins E57 and K48 after removal of the template cores. (a) CLSM image of one capsule in aqueous solution whose wall had been labeled with Alexa Fluor 488. The inset shows a low resolution image of several capsules. Images demonstrate the particulate nature of the capsules. (b,c) TEM and SEM image of one capsule. As TEM and SEM are performed in vacuum capsules collapse, indicating the absence of the template core and thus an empty cavity. Scale bars represent 1 μ m.

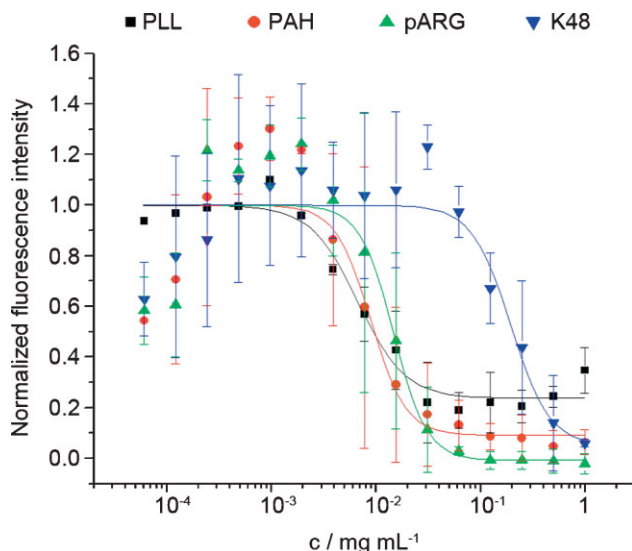


Figure 4. Comparison of the polyelectrolyte-induced toxicity on NIH/3T3 embryonic fibroblasts. Cells were incubated with the different polymers in a concentration range from 6.1×10^{-5} to $1 \text{ mg} \cdot \text{mL}^{-1}$ and some cells were left untreated as positive control for viability. Cell viability is assessed by an increase in the fluorescence signal and is given as mean of normalized intensities. The normalized fluorescent intensity is plotted against the concentration (c) and shows a sigmoidal concentration–toxicity relationship. PLL: poly(L-lysine); PAH: poly(allylamine hydrochloride); pARG: poly(L-arginine).

Conclusion

In this work we demonstrated the expression of supercharged polypeptides consisting of repetitive motifs. As a result of incorporating a single charge per almost every repeat, unfolded polyelectrolytes were obtained that are perfectly defined regarding their number and distribution of charges, monomer composition, stereochemistry, and dispersity, which is almost impossible by conventional polymerization techniques. With K48 for example, 1 positive charge per 5.7 amino acids was reached. ELPs with charged amino acids as guest residues have already been produced, but with much lower charge densities than reported herein.^[16] So far only a single-folded protein, green fluorescent protein (GFP), was equipped with similar amounts and densities of charges.^[29] However, within GFP the charges were by far not as equally distributed as in the ELP backbone. In this respect we demonstrated that the concept of supercharging by genetic engineering can be extended to other peptide-based biopolymers. When comparing supercharged ELPs with naturally occurring polypeptides, we see that with K48 it was even possible to realize a charge density comparable to only a few naturally occurring unfolded proteins,^[29,30] all while using a minimal set of the amino acid alphabet.

It was further shown that the high number of charges of these de novo designed proteins could be exploited for the fabrication of supramolecular structures. Oppositely charged variants were transformed into multilayer capsules by electrostatic interactions employing the widely used LbL technique. Due to their low toxicity, supercharged proteins like the highly charged ELPs presented in this work promise to be a favorable alternative to their chemically synthesized counterparts in the context of biomedical scaffolds. In the future we will employ supercharged ELPs for the generation of other electrostatically induced polymeric architectures such as polyplexes and hydrogels with well defined network porosity.

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